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SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

ANNUAL REPORT

ARTHUR M. DANNENBERG, JR., M.D., Ph.D.

MARCH 1, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7040

The Johns Hopkins University
School of Hygiene and Public Health
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Baltimore, Maryland 21205-2179

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: unclassified	1b. RESTRICTIVE MARKINGS: n.a.
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution authorized to U.S. Government agencies only; Specific Authority, May 5, 1989
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: The Johns Hopkins University School of Hygiene & Pub.Hlth. / 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 615 North Wolfe Street, Baltimore, Maryland 21205-2179	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command / 8b. OFFICE SYMBOL (If applicable): SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-87-C-7040
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M16 2734A875; TASK NO. AA; WORK UNIT ACCESSION NO. 377

11. TITLE (Include Security Classification): SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

12. PERSONAL AUTHOR(S): Dannenberg, Arthur M., Jr., M.D., Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 02/17/88 TO 02/16/89	March 1, 1989	40

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Sulfur mustard; human skin lesions; histology & histochemistry; inflammatory mediators; organ culture; viability of human skin at 4 C; paranuclear vacuolization test for (cont'd)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In this research program, we are (a) developing an in vitro human skin model with which to assess the amount of injury produced by topically applied sulfur mustard (SM), and (b) identifying markers of cell death and early mediators of the inflammatory response produced by SM in human skin. This second Annual Report will cover our progress since our first Annual Report, i.e., from February 17, 1988 to February 16, 1989.

The most promising measure of skin injury is our paranuclear vacuolization test. SM was applied in various dilutions to full-thickness 1.0-cm^2 human skin explants. The explants were incubated at 37 C for 24 hr, histologic sections were made, and the number of vacuoles was determined microscopically. We have now studied these vacuoles in more depth. Electron microscopy has shown that there are two types of paranuclear vacuoles: the toxicant type and the storage type. Both types of vacuoles are readily discernible by light microscopy. Only the toxicant type is produced by the application

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code): 301-663-7325 / 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

18. SUBJECT TERMS (continued from page 1 of DD Form 1473)

epidermal injury; acid phosphatase; trypsin-like enzymes; deoxyribonuclease, ribonuclease; hydroxyproline; leukotriene B_4; interleukin 1.

19. ABSTRACT (continued from page 1 of DD FORM 1473

of SM. It consists of a unilateral blebbing of the nuclear membrane accompanied by clumping of the chromatin. The storage type of vacuole seems to be due to an autolytic phenomenon in which the contents of the nucleus and its membrane are digested. The paranuclear vacuolization test for injury to human skin by sulfur mustard is by far the most convenient and practical way to assess the efficacy of protective ointments and decontaminating agents. It could be readily automated in a computerized image analyzer.

Numerous assays were made this year in an attempt to find markers of cell death and early inflammatory mediators in first-day culture fluids of human skin explants to which SM was topically applied. These included assays for ribonuclease, deoxyribonuclease, interleukin 1, various eicosanoids, acid phosphatase, trypsin-like enzymes, and hydroxyproline, a product of collagenase action. In each case, the culture fluids from both SM-treated explants and control explants contained similar amounts of these substances. Therefore, these inflammatory mediators cannot be used as markers of injury produced by SM. The only useful biochemical tests found to date were the test for plasminogen activator by the ^{125}I-fibrin plate method and the test for protein synthesis by measuring ^{14}C-leucine incorporation.

Attempts to identify other phlogistic factors, associated with human skin injury by SM, will be made during the remaining part of our contract.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985)

SUMMARY

In this research program, we are (a) developing an *in vitro* human skin model with which to assess the amount of injury produced by topically applied sulfur mustard (SM), and (b) identifying markers of cell death and early mediators of the inflammatory response produced by SM in human skin. This second Annual Report will cover our progress since our first Annual Report, i.e., from February 17, 1988 to February 16, 1989.

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INTRODUCTION

Full-thickness explants of human skin survive well in organ culture (1). Discarded skin can be obtained from a variety of surgical procedures and/or from recent autopsies. Cultured skin explants were used to assess the toxicity of topically applied chemicals. For this purpose, our paranuclear vacuolization test (1) has proven to be most practical; our [^{14}C]leucine incorporation test (1), while more time-consuming, is quite satisfactory.

This report describes further progress in the studies presented in our first Annual Report (1a), especially the studies on paranuclear vacuoles. It also describes a number of new types of experiments representing our efforts to find a simple biochemical test for SM injury to full-thickness human skin explants.

The purpose of all these studies is to develop methods for assessing the degree of SM injury to human skin in vitro, so that prophylactic ointments and decontaminating procedures can be screened.

MATERIALS AND METHODS

Organ culture of 1.0-cm^2 skin specimens (1)

The skin specimens were placed on a sterile plastic sheet, made wet with Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY, Cat. No. 310-4025) containing penicillin (1000 U/ml) and streptomycin (1000 ug/ml), and the subcutaneous fat was removed with scissors. Gloves and a surgical mask were worn, and these procedures were performed in a hood.

The skin was cut precisely into 1.0-cm^2 full-thickness pieces and washed three times with the antibiotic-containing Hanks' solution. Then, each explant was placed in a small, sterile, plastic Petri dish (35 x 10 mm, Falcon Plastics, Division of Becton Dickinson Co., Oxnard, CA). The epidermis was patted dry with sterile surgical gauze, and two drops of RPMI 1640 culture medium was added to the Petri dish in order to keep the dermal (underneath) side of the explant moist. Then, in a stainless steel hood with a face draft of 150 linear feet of air per minute, we spread (by means of a Hamilton syringe) 10 ul of dilute SM (or its vehicle) over the entire (dry) upper surface of the explant. Our standard concentration of SM was 1.0% in methylene chloride, but 0.2% was also used for some of the studies herein reported. The specimens were left 30-40 min at room temperature in the hood before they were organ-cultured.

Our culture medium (2.0 ml/Petri dish) was composed of medium RPMI 1640 containing glutamine (GIBCO Laboratories, Cat. No. 320-1875), supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and additional glutamine (2.0 mM). (The final concentrations are in parentheses.) For the [^{14}C]leucine incorporation experiments, [^{14}C]leucine (0.25 uCi/ml, 350 mCi/mM) was included. When hydroxyproline was to be assayed in the culture fluids, Dulbecco's Modified Eagle Medium (GIBCO, Cat. No. 320-1885) was substituted for the RPMI medium because the former does not contain OH-proline.

Three small Petri dishes, each containing an explant, were then placed in one large Petri dish (100 x 15 mm, Falcon Plastics), and the large Petri dishes were stacked in a heavy plastic vacuum jar (Oxoid U.S.A., Columbia, MD). The jar was gassed with a 95% O_2 + 5% CO_2 mixture at 1.1-1.2 atmospheres of pressure, and the jar was sealed. It was rocked six times per minute in an incubator at 36 C for 24 hr. The tops of the 1.0-cm^2 skin explants were not covered by the culture medium, but were exposed directly to the gaseous O_2/CO_2 mixture. They did, however, become moist (see 1).

Preparation of 1- to 2-um glycol-methacrylate-embedded tissue sections (1-3)

Water-soluble methacrylate. The JB-4 kit (Polysciences, Inc., Warrington, PA) was used. The kit contains three components: Solution A (glycol methacrylate, modified with 2-butoxyethanol), Solution B (the accelerator, N,N-dimethylaniline in polyethylene glycol 400), and Catalyst C (benzoyl peroxide). Directions for their use are provided with each kit.

Fixation. A 10% buffered formalin was obtained from Columbia Diagnostics, Springfield, VA. This product contains 3.7-4.0% technical grade formaldehyde, 18.6 g/L monobasic sodium phosphate, 4.2 g/L sodium hydroxide, and about 1% methanol. It has a pH of 7.2 and a buffer osmolality of 290 mOsm/kg. A modified Karnovsky's formulation (4) was prepared by mixing 100 ml of 50% glutaraldehyde (Polysciences, Inc.), 25 ml of glycerol, and 900 ml of the 10% buffered formalin. The 1.0-cm^2 skin explants were placed in this fixative at 4 C for 1 or 2 days.

Embedding (2). The fixed tissue samples were washed at 4 C for 24 hr in 0.05 M potassium phosphate buffer, pH 7.2. (The samples may be left in this cold buffer for several days.) They were dehydrated for 2 hr each in 50%, 70%, 95%, and 95% ethanol (all containing 2.5% glycerol), after which they were infiltrated 1-3 days at 4 C in Solution A with catalyst added.

A molding tray containing multiple 6 x 5 x 12 mm wells (Polysciences) was placed in a shallow basin of cracked ice. The bottom of each well was covered with catalyzed Solution A containing 5% Solution B (i.e., a 1:20 ratio of B:A), prepared with cold (4 C) reagents. Then each Solution-A-infiltrated explant was cut through its center (perpendicular to its epidermal surface) and its cut surface was carefully placed flat against the bottom of a well in the molding tray. The wells were filled with the catalyzed Solution A and B mixture (described above), and aluminum JB-4 block holders (Polaron Instruments, Inc., Division of BioRad, Cambridge, MA) were placed on the top of each well. Slight overfilling of the wells eliminated the need for special measures to exclude oxygen. The entire basin was placed in a 4 C refrigerator, and the glycol methacrylate in the molding tray was allowed to polymerize overnight. Then the blocks were removed from the tray and examined for hardness. If too soft, they were placed in a desiccator at room temperature until adequately hardened.

Coating microscope slides. Microscope slides were sprayed lightly (in a hood) with a fine mist of Solution A. Care was taken to avoid coalescence of the droplets on the slides. They were promptly placed in an oven at about 80 C and dried (usually overnight). (Note: The noxious vapors released during drying should not be inhaled.) Then, this spraying-drying sequence was repeated.

Sectioning. The JB-4 microtome (Du Pont Instruments, Norwalk, CT) was used with Ralph-type glass knives. The knives were made on a Histo-Knife-maker (LKB Instruments, Rockville, MD) from 38-mm glass strips, broken 3-4 mm from the score at an intermediate fulcrum setting (1).

The blocks were faced until the full width of tissue was just encountered. Sections were then cut at 2 um. Each section was straightened as much as possible while on the knife, transferred to an elliptical pool of water on a precoated slide, and allowed to spread. Then the water was carefully blotted away, and the slide was dried at room temperature. (Sections spread better when the water contains about 1% ammonium hydroxide.)

Staining. Giemsa staining was prepared as follows (2). Ten milliliters of 0.2 M Tris-maleate buffer was diluted with 40 ml of water and adjusted to pH 5.4 with 1 N NaOH. Five milliliters of Giemsa concentrate (Harleco, Cat. No. 620, Gibbstown, NJ) was added, and the mixture was stirred vigorously for at least 10 min. The stain was then filtered two times into a standard Coplin jar, each time through fresh filter paper (Grade 588 for coarse precipitates, Schleicher and Schuell, Inc., Keene, NH). Fresh working stain was prepared daily. The Giemsa concentrate (kept at room temperature) could be used satisfactorily for at least 6 months.

The plastic sections were stained in the Coplin jar for 60 min, rinsed briefly in a jar of distilled water, dried immediately in a warm airstream, and mounted under a coverslip in a methacrylate medium (Flo-Texx, Lerner Laboratories, Stamford, CT).

Glycol-methacrylate-embedded tissue sections are easier to prepare than paraffin-embedded sections. Glycol methacrylate (GMA) is more hydrophilic than paraffin. Therefore, tissue specimens do not need to be completely dehydrated for embedding, nor do the resulting sections need to be rehydrated before staining.

Counting paranuclear vacuoles. All paranuclear vacuoles seen microscopically with a 40 X objective lens and a 10 X eye piece were counted in the entire epidermis of the 1.0-cm explant. A photograph of ten such vacuoles appears in reference 1. The counts presented in this Second Annual Report were made by a different postdoctoral fellow from the one who made the counts for our First Annual Report, March 1988 (1a). See Discussion.

Electron microscopy

We used standard electron microscopy techniques (5). Specifically, small (2-to 3-mm) central pieces of the skin explants were fixed for 4 hr in cold (4 C) 2% paraformaldehyde-2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), containing 0.3 M sucrose. They were then transferred to this same sucrose-containing buffer (without the aldehydes) and left overnight at 4 C. They were fixed in 1.0% osmium tetroxide in sucrose-free buffer for 90 min, rinsed in the buffered sucrose solution, dehydrated in alcohols, embedded in Spurr's epoxy formulation (6), cut on an ultramicrotome, and stained with lead citrate (7).

Viability of the explants

Histologic evaluation is one of the best ways to determine cell and tissue viability in organ-cultured explants (personal communication of the late Dame Honor B. Fell, Strangeways Laboratories, Cambridge, England). The human skin explants survived well in organ culture for at least 7 days, and there was no morphologic evidence of epidermal cell death in normal skin explants.

Acid phosphatase released in vitro by full-thickness human skin explants

Culture fluids from 1.0-cm^2 full-thickness human skin explants (exposed in vitro to SM) were assayed for acid phosphatase by the following method, which was adapted from Bergmeyer, et al. (8):

To each 1.2 X 10.0 cm standardized colorimeter tube (placed in cracked ice), were added 0.50 ml of 0.09 M sodium citrate buffer (pH 4.8), 0.50 ml of 15.2 mM disodium p-nitrophenyl phosphate (phosphatase substrate, Sigma Chemical Co., St. Louis, MO, Cat. No. N-3002) in the same buffer, and 0.20 or 0.50 ml of a given culture fluid. The tubes were incubated for 30 min or 20 hr in a water bath at 36 C, and the reaction was stopped by placing the tubes in water containing cracked ice. Immediately before the optical density (OD) of each tube was read at 410 nm, the tubes were removed from the ice, and 2 ml of 0.10 N NaOH at 23 C was added. The OD's were read in a Beckman DB-G spectrophotometer against a reagent blank containing incubated substrate and culture medium.

A trypsin-like enzyme released in vitro by full-thickness human skin explants

Culture fluids from 1.0-cm^2 full-thickness human skin explants, exposed in vitro to SM, were assayed for trypsin-like proteases with the synthetic peptide substrate, t-butyl-oxycarbonyl (BOC)-L-leucyl-glycyl-L-arginyl-7-amino4-trifluoromethylcoumarin (LGA-AFC) (9).

This substrate was obtained from Enzyme Systems Products, (P.O. Box 2033, Livermore, CA 94550, Cat. No. 58-AFC). It was dissolved in dimethyl formamide (DMF) to a concentration of 20 mM. Fifty microliters of the AFC substrate, 900 ul of 0.05 M TES buffer (pH 8.2), and 50 ul of the explant culture fluid were incubated for 24 hr at 37 C. TES designates N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (Sigma, Cat. No. T-1375). The fluorescence of the incubated solutions was read at 0 and 24 hr in an AMINCO-BOWMAN (American Instrument Co., Inc., Silver Spring, MD) spectrofluorometer with an excitation wavelength of 400 nm, and an emission wavelength of 505 nm. As a control, the substrate solution without enzyme (50 ul) was incubated for 24 hr in 950 ul of the TES buffer, and the increase in its fluorescence was subtracted from the increase in the fluorescence produced by the lesion culture fluids. The pH of the incubated solutions was usually 8.1.

The AFC standard (Sigma, Cat. No. A-8401) was used to calibrate our assay system. Little or no spontaneous hydrolysis of LGA-AFC occurred in the pH range 5.0-9.0.

Deoxyribonuclease (DNase) released in vitro by full-thickness human skin explants

DNase in 24-hour culture fluids from full-thickness 1.0-cm^2 human skin explants was assayed by the following method (10): The culture fluids (1.5 ml) were mixed with 2.0 ml of sodium phosphate buffer (0.05 M, pH 6.2) and 0.1 ml of 0.25 M $MgSO_4$ and placed in a cracked ice bath. DNA (deoxyribonucleic acid [sodium salt], type III from salmon testes, Sigma, Cat. No. D-1626) (2.5 mg) in 1.0 ml of 0.9% NaCl was chilled in the ice bath and added to the buffered culture fluids. The pH was determined and adjusted to 6.0-6.3 with 1.0 N NaOH. Then 2.0 ml was removed as a 0-hour control, and 2.0 ml was incubated for 2 (or more) hours at 38 C in a shaking water bath. The reaction was stopped at 0 hour and 2 (or more) hours by adding 2.0 ml of cold $LaCl_3$ precipitating reagent. The precipitated 0- and 2-hr samples were centrifuged at 4 C for 20 min at 8000 rpm (4000 g). Then, the supernates were removed and read in 1-cm cuvettes in a Beckman DB-G spectrophotometer at 260 mu.

The $LaCl_3$ reagent was prepared weekly as follows: 80 ml of 12 N HCl, 120 ml of water, and 5.0 gm of $LaCl_3$ in sufficient 95% ethanol (diluted from 100% ethanol) to make 1000 ml.

With both the DNase (and RNase) assays, the approximate pH optimum and a satisfactory incubation time were determined with explant culture fluids.

Ribonuclease (RNase) released in vitro by full-thickness human skin explants

The RNase in 24-hour culture fluids from full-thickness 1.0-cm^2 human skin explants was assayed by the following method (10). The culture fluids (1.5 ml) were mixed with 2.5 ml of 0.05 M sodium phosphate buffer (pH 6.2) and 0.1 ml of 0.25 M $MgSO_4$ and placed into a cracked ice bath. RNA (ribonucleic acid, type XI from yeast, Sigma, Cat. No. R-6750) (1.0 mg) in 1.0 ml of 0.9% NaCl was mixed with 2.0 ml of the phosphate buffer, chilled in the ice bath, and added to the buffered culture fluids. The pH was taken and adjusted to 6.0-6.25 with acid or alkali. Then, 2.0 ml was removed as the 0-hour control, and 2.0 ml was incubated for 2 or more hours at 38 C in a shaking water bath. Cold $LaCl_3$ precipitating reagent (2.0 ml) was added to both specimens. This $LaCl_3$ reagent was prepared with 40% ethanol instead of the 95% employed in the DNase assay. The rest of the RNase assay was the same as that described for DNase above.

Hydroxyproline released in vitro by full-thickness human skin explants

Hydroxyproline is almost unique to collagen, in that few other proteins contain this amino acid (11). The release of OH-proline-containing peptides into the culture fluids can, therefore, be used as a measure of collagen breakdown within the organ-cultured explants.

The culture fluids (1.0 ml) were mixed with 12 N HCl (1.0 ml) in screw-capped tubes with Teflon seals, and heated in an oven at 105 C for 18 hr. Two ml of 6N NaOH was added to bring the pH between 6.5 and 7.5. The samples were then frozen until they were assayed for OH-proline.

OH-proline assay. Chloramine-T reagent (1.0 ml) was added to each sample. This reagent contained 2.82 g of chloramine-T (Sigma, Cat. No. C-9887) in 50.0 ml of 2-methoxyethanol (methyl cellosolve). The reaction was allowed to proceed for exactly 25 min with repeated vortexing. Then, 3.6 M sodium thiosulfate (3.0 ml) was added, and the tubes were vortexed again. After toluene (5.0 ml) was added, the tubes were vortexed once more. The toluene layer, which extracted interfering substances, was removed by centrifugation, and discarded. Then, the tubes were capped, heated for 30 min in a boiling water bath, and cooled. Heating in the presence of chloramine-T and thiosulfate oxidizes OH-proline to pyrrole.

After the toluene (5.0 ml) was replaced, the tubes were shaken for 4 min and recentrifuged. The toluene layers (2.5 ml) were placed into colorimeter tubes and mixed with 1.0 ml of a solution containing Ehrlich's reagent. After exactly 25 min at room temperature, the optical densities were read in a spectrophotometer at 560 nm.

The Ehrlich's reagent solution contained 120 g of p-dimethylaminobenzaldehyde (Sigma, Cat. No. D2004), 1200 ml of ethanol, and 27.4 ml of concentrated sulfuric acid. It reacted with the pyrrole to form a chromaphore.

Standard solutions containing 5 ug and 10 ug of OH-proline per ml were included in each assay as positive controls. They had optical densities of 0.250 and 0.500, respectively.

Leukotriene B_4 and other eicosanoids released in vitro by full-thickness human skin explants

The human skin explants (1 cm^2) were incubated with 3 uM ^{14}C-labeled arachidonic acid, specific activity 390 mCi/mM, (Du Pont NEN Research Products, Boston, MA, Cat. No. NEC-756) for 17 hr at 36 C in our standard RPMI 1640 culture medium. They were washed with cold medium; then, SM (1.0%) or $MeCl_2$ was applied, and the explants were incubated an additional 3 hr. The arachidonic acid metabolites in the culture fluids were analyzed by high performance liquid chromatography with various concentrations of acetonitrile in 0.1% acetic acid as the solvents.

Interleukin 1 (IL-1) released in vitro by full-thickness skin explants

The culture fluids from SM-exposed and control full-thickness human (and rabbit) skin explants were assayed for IL-1 in Dr. Gail S. Habicht's laboratory at the State University of New York at Stony Brook by the thymocyte proliferation assay (12). Single cell suspensions of thymocytes from 4- to 8-week-old BALB/c or C3H/HeJ mice were prepared by gently teasing the thymus apart with sterile 18-gauge needles followed by gentle agitation with a sterile Pasteur pipette. Aggregates were removed by a 10-min gravity sedimentation. The cells were washed once with Hanks' balanced salt solution and resuspended to a density of 5×10^6 cell/ml in culture medium RPMI 1640 containing 10% calf serum, 1% antibiotics, 1% nonessential amino acids, 1% glutamine, and 5×10^{-5} M 2-mercaptoethanol. The thymocytes were cultured for 72 hr at 5×10^5 cells/well in Falcon 96-well, flat-bottomed tissue culture plates in the presence or absence of 0.3 ug/ml Con A. Various dilutions of the samples to be assayed for IL-1 activity were added. The cultures were

pulsed with 1 uCi ^{3}H-thymidine (^{3}H-TdR, 6.7 Ci/mmol, Du Pont NEN Research Products, Cat. No. NET-027) for the final 8 hr of incubation. The cells were then collected on glass fiber filter paper with an automatic harvester (Otto Hiller, Madison, WI). Each sample was added to 4 ml scintillation fluid (National Diagnostics, Freehold, NJ) and counted in a well-type beta-scintillation counter (Mark III, Searle Analytical, Des Plains, IL). The results were expressed as the increase in disintegrations per minute (dpm). This increase represents the ^{3}H-TdR incorporation due to IL-1, i.e., the dpm of the thymocyte preparation containing Con A plus the culture fluid sample minus the dpm of the thymocyte preparation containing only Con A.

RESULTS

Paranuclear vacuolization in human skin exposed to SM

The number of paranuclear vacuoles in the epidermis can be used as a measure of the toxicity of SM, following its topical application to full-thickness human skin explants (1). When studied by transmission electron microscopy, these vacuoles proved to be of two types: the "storage type" and the "toxicant type" (Figures 1, 2, and 3). Storage-type vacuoles usually had a perinuclear configuration, i.e., they formed a halo around the nucleus. They seemed to be associated with a disintegration of the nuclear membrane and dispersion with partial autolysis of the nuclear chromatin. In contrast, toxicant-type vacuoles usually were found on one side of the nucleus and often indented it. The nuclear membrane was present (although it was sometimes broken) and the chromatin was usually clumped and condensed. These characteristics enabled us to differentiate between the two types of vacuoles by light microscopy under a 40 X objective lens.

We therefore recounted the paranuclear vacuoles in most of the series of full-thickness human skin explants analyzed for our Annual Report of March 1988 (1a), listing in separate categories storage-type vacuoles, toxicant type vacuoles, and total paranuclear vacuoles (which was the sum of both types) (Tables 1, 2 and 3). We also analyzed the vacuoles in additional human skin specimens in the same manner. Not all paranuclear vacuoles could be definitely assigned to one or the other group, but most vacuoles were usually sufficiently distinct for us to do so. The rare intermediate-type vacuole was assigned to one group or the other depending on its most prevalent characteristics.

The number of both storage-type and toxicant-type vacuoles was found to be increased in explants after 3 to 5 days in the refrigerator at 4 C, but this number did not always increase thereafter (Table 1). Incubation for 4 or 24 hr at 36 C usually decreased the number of both types of vacuoles (Table 1). Apparently, nuclei of these cells lysed and therefore could no longer be counted.

Sulfur mustard (0.2% applied topically) did not increase the number of storage-type vacuoles, but markedly increased the number of toxicant-type vacuoles (Tables 2 and 3). Preincubation of the explant in RPMI-1640 for 4 hr at 36 C had no consistent effect on the number of toxicant-type vacuoles produced by the subsequent application of 0.2% SM, followed by a 24-hr incubation at 36 C (Table 3).

Since the storage-type vacuoles do not increase after the application of SM, the counting of all types of vacuoles in the tissue section (i.e., both toxicant-type and storage-type) provides a satisfactory representation of sulfur mustard toxicity, reduces the counting time, and makes such counting easier to automate with an image analyzer.

Synergistic effects of SM and organ culture "shock" on hydroxyproline and acid phosphatase released by full-thickness human skin explants

Hydroxyproline release. In organ culture, rabbit SM lesions produced in vivo released more collagenase and proteoglycanase than was released by normal skin (11,13). We proved this by measuring the culture fluids for both of these enzymes and for hydroxyproline and glycosaminoglycans. In these experiments, second- and third-day culture fluids contained much higher levels of these enzymes and their products than did first-day culture fluids (11,13). Thus, SM and the "shock" of organ culture seem to be synergistic in causing the cells, probably fibroblasts (13,14), in the explants to break down the extracellular matrix.

Such synergism might be used to develop a sensitive test for the effects of SM on full-thickness human skin explants. We therefore exposed such explants to 1.0% SM and cultured them for 3 days. The culture fluids were changed daily. Then, the third-day culture fluids were cleared by centrifugation and assayed for hydroxyproline. OH-proline levels in the culture fluids reflect collagenase activity and are relatively easy to measure (11). Dulbecco's Modified Eagle Medium was used (instead of RPMI 1640) whenever OH-proline was assayed (11).

The third-day culture fluids from the SM-exposed explant appeared to be lower (rather than higher) in OH-proline content than their respective controls (Table 4).

Since the presence of extravasated serum (or the infiltrating cells) seemed to be necessary to enhance collagenase production in vivo, we cultured human skin explants for 2 days in Dulbecco's medium in the presence (and absence) of 10% fetal calf serum, replacing the medium daily. Then, SM was applied to half of the explants, and they were cultured for an additional 1, 2, and 3 days in serum-free Dulbecco's medium. On each of these days the medium was collected, centrifuged, and stored at -70 C until assayed for OH-proline.

Fetal calf serum had no stimulatory effect on OH-proline release into the culture fluids (Table 4). Again, as in the preliminary 3-day experiment just described, SM seemed to decrease the amount of OH-proline released by the explants. In other words, SM apparently decreased the production of collagenase by the cells in the explants. (Keratinocytes and fibroblasts are known to produce and release this enzyme.) We shall continue this type of experiment, in order to determine whether it could be used as a simple biochemical test for measuring SM toxicity to human skin in vitro.

Acid phosphatase release. First-, second-, and third-day culture fluids from SM-exposed and control human skin explants were also measured for acid phosphatase, a major lysosomal enzyme of fibroblasts, macrophages and other cells (14). However, no significant differences between the SM and control

groups were found (Table 5). In fact, no definite acid phosphatase activity was detected in culture fluids from the skin explants. The results in Table 5 seem to have been caused by variations in the spontaneous hydrolysis of the substrate during the two incubation times.

Conclusion. Thus to date, the synergism between SM and the "shock" of organ culture is limited to enzymes hydrolysing the extracellular matrix (collagen and ground substance) in SM lesions produced in vivo. This synergism was not apparent in human skin explants exposed to SM in vitro.

Enzymes hydrolyzing boc-leucyl-glycyl-arginyl aminofluorocoumarin (LGA-AFC) released by full-thickness human skin explants

One of the enzymes that can hydrolyze LGA-AFC is plasminogen activator (PA). PA was elevated in first-day culture fluids from SM-exposed human skin explants when it was assayed by the fibrin plate method (see our March 1988 Annual Report (1a)). Therefore, culture fluids from human skin explants exposed to SM were assayed with LGA-AFC in the presence and absence of plasminogen (15-17). These culture fluids were also assayed in the presence and absence of aprotinin, which is an inhibitor of plasmin, but not of plasminogen activator (see 9 and 18).

The results of these experiments are presented in Table 6. Evidently, LGA-AFC cannot be used to detect the increase in PA activity caused by SM in human skin explants. Apparently, so many other enzymes hydrolyze this substrate that PA activity is eclipsed even in the presence of PA's specific substrate, plasminogen, and in the presence of aprotinin, which inhibits several of these enzymes (but not PA).

Deoxyribonuclease (DNase) and ribonuclease (RNase) released by full-thickness human skin explants exposed to SM

SM is known to injure the DNA of epidermal cells (19,20). When such injury is irreversible, the DNA is hydrolyzed by DNases. Since the release of DNase into the culture fluids of SM-exposed human skin could prove to be a sensitive indicator of epidermal cell damage, we assayed these fluids for this enzyme.

Unfortunately, SM had no consistent effect on the release of DNase into the culture fluids nourishing the explants (Table 7). Similar results were found in the few such fluids assayed for RNase (also Table 7). Therefore the assay of these enzymes does not seem to be a promising method for assessing in vitro SM injury to human skin.

Similar studies with full-thickness rabbit skin explants exposed in vitro to nitrogen mustard were slightly more promising (Table 7). On the average, rabbit skin contained higher levels of DNase than did human skin.

Leukotriene B_4 production by full-thickness human skin explants exposed to SM

One cm^2 full-thickness explants of normal human skin were incubated with ^{14}C-arachidonate for 17 hr. About 70% of the ^{14}C-arachidonate was incorpo-

rated into the explants: Such incorporation was determined by measuring the radioactivity, both in the culture fluids and in solubilized skin explants.

After the 17 hr incubation and washing to remove the free ^{14}C-arachidonate, SM (1%), or its diluent $MeCl_2$, was topically applied. The explants were then incubated an additional 3 hr. The ^{14}C-arachidonate metabolites in the explant culture fluids were analyzed by high performance liquid chromatography by Dr. E.W. Spannhake's group in our department.

No LTB_4, nor any other labeled eicosanoid, was found. The only labeled substance was arachidonate itself. About 3% of the incorporated arachidonate was released during the 3 hr incubation period.

Cultured epidermal cells are known to synthesize eicosanoids. We did not detect them, probably because of absorption of the arachidonate or its metabolites by the collagen and ground substance in the explants or because of adsorption to the plastic culture dishes. Thus, if SM induces LTB_4 production in human skin, other procedures must be used to detect it, e.g., enzyme-linked immunoassays (ELISA). Also, it would be of interest to perform autoradiography on tissue sections of explants incubated with ^{14}C-arachidonate, and to add labeled LTB_4 to our explant cultures to test how much is destroyed or absorbed.

Interleukin 1 (IL-1) released by full-thickness human (and rabbit) skin explants exposed to SM

Interleukin 1 is a growth factor, produced by a variety of cells, including epidermal cells. Beck and *Habicht* (12) discovered that it (or a product of its action) was a chemotactic factor, equivalent in potency to leukotriene B_4 and the C5a peptide derived from the fifth component of complement. We began our collaboration with these investigators shortly after this discovery, because in SM lesions the granulocyte and macrophage infiltration caused by such chemotactic agents probably contributes to tissue damage and blister formation.

Our first experiments together (in 1986) concerned IL-1 released in organ culture by developing and healing rabbit SM lesions. IL-1 was present in the organ-culture fluids, more so in SM lesions than in controls, and more so in developing lesions than in healing lesions. In 1987, we found that full-thickness rabbit skin explants, treated with SM in vitro and cultured in the presence of mononuclear (macrophage) peritoneal exudate cells (MN), produced more IL-1 than did controls similarly cultured and treated only with the SM diluent methylene chloride. These studies suggested that the SM-injured epidermis produces a factor that stimulates resident macrophages and fibroblasts to secrete IL-1. The IL-1, in turn, would initiate the inflammatory process.

This year we performed these promising experiments again, but, unfortunately, were not able to reproduce them (Table 8). For unknown reasons, the IL-1 activity in the various culture fluids was quite variable. Culture fluids from human skin exposed in vitro to SM were also assayed along with controls. No consistent differences between them were found (Table 9).

Thus, to date we have shown that both human and rabbit full-thickness skin explants produce IL-1, but SM does not increase the IL-1 production.

DISCUSSION

Apoptosis is a programmed cell death that does not result in inflammation (21). It is the fate of isolated senile cells in normal tissues, unneeded cells during embryological development, and cells in involuting tissues, e.g., those in the uterus after parturition. It also occurs in cells after ultraviolet and X-radiation and after the administration of radiomimetic drugs. SM is such a drug, and the clumping and condensation of the nuclear chromatin that we observed also occur in cells undergoing apoptosis. However, other characteristics of apoptosis (e.g., plasmalemma blebbing) were not observed; and SM always produces an inflammatory reaction. Thus, the nucleic acid damage produced by SM seems to initiate the apoptotic process, and then necrosis of the entire cell seems to follow, interrupting the programmed cell death. The toxicant type of paranuclear vacuole produced by SM has not, to our knowledge, been described in the apoptotic literature, perhaps because cells of the intact epidermis have not been carefully studied for apoptotic phenomena.

Table 10 presents a comparison of the counts obtained by two different investigators counting paranuclear vacuoles in the epidermis of the same tissue sections under the microscope. In general, variations from 50% to 250% exist. Part of these variations may be due to choosing a different section to count: Two sections of the same tissue are usually placed on one slide, but only one is generally counted. Unfortunately, since we did not expect to count the sections twice, we did not note which section was counted in each case. (We undertook recounting only after the two types of vacuoles, storage-type and toxicant-type, were discovered.) Part of the variations may be due to the choices made by each investigator on whether or not to include very small vacuoles, and whether or not to include overtly pyknotic cells with vacuoles. Histological quantitation always shows some investigator variation. It is most accurate when the same investigator counts all of the slides from the entire experiment. In that case, a straight-line dose-response curve can be obtained (see references 1 and 1a).

CONCLUSIONS

During the second year of this contract, we further developed our paranuclear vacuolization test, so that it can be used with more insight to test the efficacy of protective ointments (and decontaminants) on human skin exposed to SM.

We also evaluated a variety the early inflammatory mediators which may be altered by the topical application of SM to full-thickness human skin explants. In our first experiments, SM treatment seemed to increase the amount of interleukin 1, deoxyribonuclease, and ribonuclease in the culture fluids, but these results were not confirmed by subsequent experiments. To date, the most important mediator (increased by the application of SM) was found to be plasminogen activator, a proteolytic enzyme associated with blister formation (see our Annual Report of March 1988 (1a)).

PLANS FOR THE FUTURE

During this quarter, we submitted a research proposal for continuation of our work with sulfur mustard after our present contract expires on August 16, 1989. It concerned blister formation produced in vitro in human skin, and the role of various cytokines and growth factors in SM lesions (produced in vivo in the skin of rabbits). It also included histochemical studies on the role of reactive oxygen intermediates in such in vivo produced injury.

The time remaining in our present contract will be spent completing our studies on paranuclear vacuoles and on the early mediators of inflammation produced in full-thickness human skin explants. The latter includes (a) the C5a assays being performed by Janet Wagner and Dr. Richard DiScipio in Dr. Tony E. Hugli's laboratory in La Jolla, CA; (b) an immunocytochemical test to identify poly(ADP-ribose) in the paranuclear vacuoles with specific antibodies sent to us by Dr. Kunihiro Ueda in Kyoto, Japan; (c) assays for DNase and RNase in SM- and $MeCl_2$-exposed human epidermis removed by trypsin from the explant; (d) in vitro experiments on PMN infiltration into full-thickness rabbit skin explants; (e) the measurement (in Boyden chambers) of chemotactic activity for granulocytes and macrophages in culture fluids from SM-exposed and control full-thickness human skin explants; and (f) further studies on the decrease in OH-proline released by human skin explants exposed to SM or $MeCl_2$. Then, we shall prepare our final report and one or two manuscripts for publication.

Figure 1: Full-thickness human skin explant exposed topically to 1% SM and cultured for 48 hr. Depicted is a typical toxicant-type paranuclear vacuole indenting one side of the nucleus. The nuclear chromatin is clumped and condensed. The nuclear membrane is mostly intact. Explants cultured for 24 hr show similar vacuoles. X 22,700.

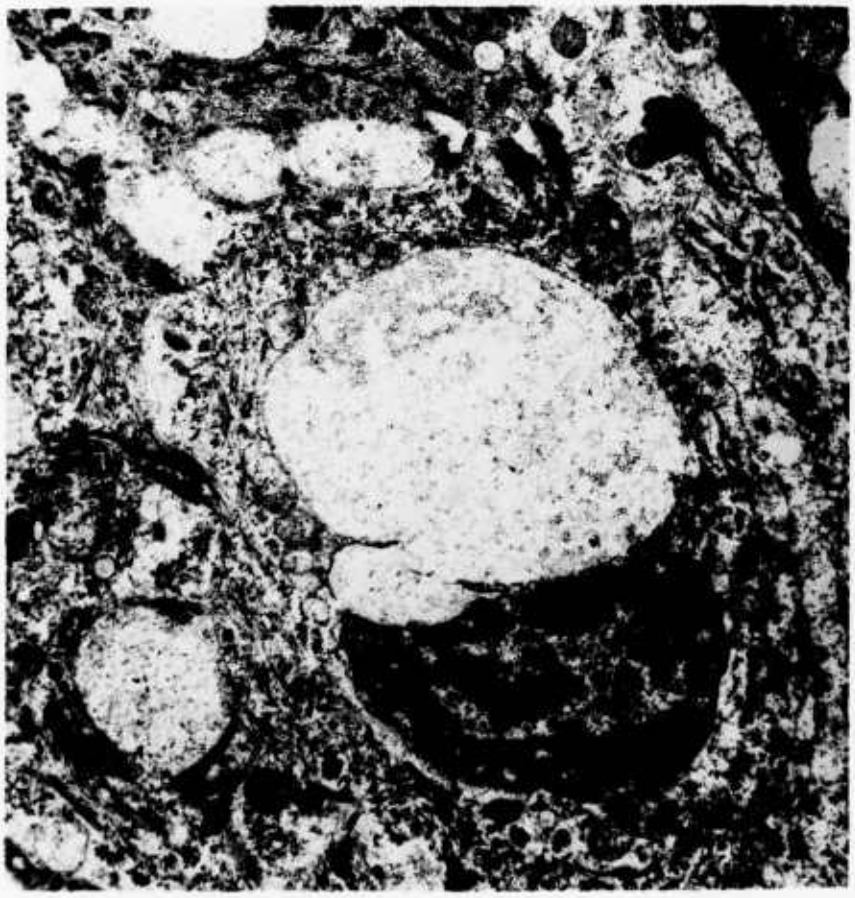

Figure 2: Full-thickness human skin explant not exposed to SM and not incubated (similar to those found after incubation at 24 and 48 hr). Depicted is a normal-appearing nucleus with normally distributed chromatin. X 20,000.

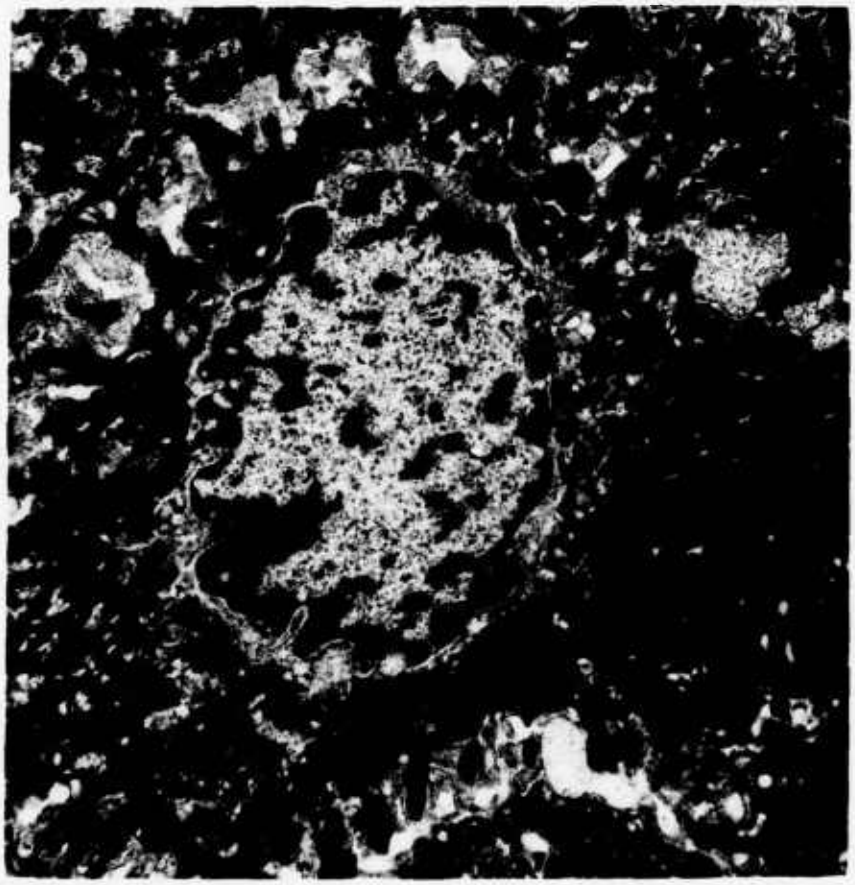

Figure 3: Full-thickness human skin explant stored at 4 C for 6 days. The nuclear chromatin is evenly dispersed and partly digested. The nuclear membrane is no longer intact. A storage-type paranuclear vacuole surrounds the disintegrating nucleus. X 21,100.

Table 1: Effect of storage of normal full-thickness human skin explants at 4 C and of subsequent 4-hr or 24-hr incubation at 36 C on the number of paranuclear vacuoles in the epidermal cells

Skin Sample No. and type of skin	Days in storage at 4 C	Number of paranuclear vacuoles in a 1.0-cm tissue section across the untreated explant								
		at 0 hr			after 4-hr incubation			after 24-hr incubation		
		storage type	toxicant type	total	storage type	toxicant type	total	storage type	toxicant type	total
1										
Normal breast	1 day(s)	5	2	7	29	10	39	—	—	—
skin, black	4 "	67	17	84	3	1	4	13	2	15
female, age 30	7 "	72	28	100	2	4	6	13	10	23
2										
Normal breast	0 day(s)	1	1	2	2	1	3	45	14	59
skin, white	5 "	83	180	263	4	36	40	63	28	91
female, age 27	9 "	92	66	158	17	14	31	28	13	41
3	2 days	14	103	117	3	1	4	49	16	65
Normal breast	5 "	110	98	208	3	12	15	40	9	49
skin,	8 "	—	—	—	16	77	93	15	10	25
black female,	12 "	92	210	302	35	183	218	87	240	327
age 24	15 "	78	119	197	21	63	84	15	123	138
4										
Normal leg skin,	3 days	15	4	19	—	—	—	20	11	31
white female,	6 "	87	59	146	—	—	—	20	10	30
age 79	10 "	138	197	335	—	—	—	26	59	85
5	1 day(s)	2	2	4	2	2	4	9	4	13
Normal leg skin,	4 "	23	46	69	9	5	14	24	4	28
white male,	8 "	59	129	188	24	155	179	57	115	172
age 72	15 "	116	143	259	72	70	142	172	58	230
9a										
Normal arm	7 days	350	360	710	—	—	—	29	34	63
skin, white	14 "	500	250	750	—	—	—	270	176	446
male, age 30										
9b										
Normal breast	0 day(s)	31	12	43	—	—	—	25	22	47
skin, white	6 "	420	390	810	74	250	324	34	49	83
female, age 51	12 "	510	300	810	—	—	—	230	360	590
10										
Normal thigh	7 days	191	380	571	—	—	—	18	55	73
skin, black	12 "	151	510	661	—	—	—	58	230	288
male, age 79	18 "	194	310	504	—	—	—	58	190	248

Table 1: Effect of storage of normal full-thickness human skin explants at 4 C and of subsequent 4-hr or 24-hr incubation at 36 C on the number of paranuclear vacuoles in the epidermal cells (continued)

The skin was received in our laboratory 2-4 hr after surgery. It was kept refrigerated at 4 C until incubated for 4 or 24 hr at 36 C. Two explants were cultured for each value listed, and the paranuclear vacuoles were counted in two tissue sections prepared from each explant. The means of these four paranuclear counts were then calculated. The standard errors of these means were usually between 5% and 15% of their respective means.

These data show that incubation for 4 or 24 hr decreased the number of paranuclear vacuoles in skin specimens stored at 4 C for 4-15 days.

Table 2: Effect of storage of full-thickness human skin explants at 4 C on the number of epidermal paranuclear vacuoles produced by the topical application of SM

Skin sample no. and type of skin	Days in storage at 4 C	Number of paranuclear vacuoles in a central 1.0-cm tissue section across the explant after 24-hr incubation at 36 C					
		Controls			0.2% SM		
		storage type	toxicant type	total	storage type	toxicant type	total
1							
Normal breast	1 day(s)	--	--	--	28	710	738
skin, black	4 "	13	2	15	28	1310	1338
female, age 30	7 "	13	10	23	28	1040	1068
2							
Normal breast	0 day(s)	45	14	59	35	780	815
skin, white	5 "	63	28	91	41	470	511
female, age 27	9 "	28	13	41	56	910	966
3	2 days	49	16	65	49	910	959
Normal breast	5 "	40	9	49	56	1060	1116
skin, black	8 "	15	10	25	29	1030	1059
female, age 24	12 "	87	240	327	40	1010	1050
	15 "	15	123	138	30	870	900
4							
Normal leg	3 days	20	11	31	21	260	281
skin, white	6 "	20	10	30	20	400	420
female, age 79	10 "	26	59	85	28	220	248
5	1 day(s)	9	4	13	27	161	188
Normal leg	4 "	24	4	28	44	340	384
skin, white	8 "	57	115	172	41	187	228
male, age 72	15 "	172	58	230	124	168	292
9a							
Normal arm	7 days	29	34	63	78	370	448
skin, white	14 "	270	176	446	240	270	510
male, age 30							
9b							
Normal breast	0 day(s)	25	22	47	46	330	376
skin, white	6 "	34	49	83	60	420	480
female, age 51	12 "	230	360	590	350	500	850
10							
Normal thigh	7 days	18	55	73	--	--	--
skin, black	12 "	58	230	288	--	--	--
male, age 79	18 "	58	190	248	--	--	--

Table 2: Effect of storage of full-thickness human skin explants at 4 C on the number of epidermal paranuclear vacuoles produced by the topical application of SM (continued)

SM (0.2% in $MeCl_2$) was topically applied to the stored full-thickness skin specimens within an hour of their removal from the refrigerator. The controls received no topical application. Two explants were cultured for each value listed, and the paranuclear vacuoles were counted in two tissue sections prepared from each explant. The means of these four paranuclear counts were then calculated. The standard errors of these means were usually between 5% and 15% of their respective means.

These data show that 0.2% SM increased the total number of paranuclear vacuoles, both in fresh skin specimens and in those stored at 4 C. This increase was solely due to the increased number of toxicant-type vacuoles. The number of storage-type vacuoles was not increased by SM.

Table 3: Effect of 4-hr preincubation of stored full-thickness human skin explants on the toxicity of SM applied subsequently

Skin sample no. and type of skin	Days in storage at 4 C	Number of paranuclear vacuoles in a 1.0-cm tissue section across the explant that was topically exposed to 0.2% SM					
		No pre-incubation			After 4-hr pre-incubation at 36 C		
		storage type	toxicant type	total	storage type	toxicant type	total
1 Normal breast skin, black female, age 30	4 days	28	1310	1338	44	1050	1094
	7 "	28	1040	1068	16	410	426
2 Normal breast skin, white female, age 27	5 days	41	470	511	29	650	679
	9 "	56	910	966	47	1250	1297
3 Normal breast skin, black female, age 24	2 "	49	910	959	37	900	937
	5 "	56	1060	1116	57	940	997
	8 "	29	1030	1059	33	790	823
	12 "	40	1010	1050	60	780	840
	15 "	30	870	900	7	790	827
5 Normal leg skin, white male, age 72	4 days	44	340	384	60	400	460
	8 "	41	187	228	68	129	197
	15 "	124	168	292	168	178	346
9b Normal breast skin, white female, age 51	0 day(s)	46	330	376	--	--	--
	6 "	60	420	480	83	780	863
	12 "	350	500	850	250	410	660

SM (0.2%) was applied to both the preincubated and control skin explants. Then, they were incubated at 36 C for 24 hr, and the paranuclear vacuoles were counted in two tissue sections prepared from each explant. The explants that were not preincubated for 4 hr were kept at 4 C during that time. Two explants were cultured for each value listed, and the paranuclear vacuoles were counted in two tissue sections prepared from each explant. The means of these four paranuclear counts were then calculated. The standard errors of these means were usually between 5% and 15% of their respective means.

These data show that preincubation of stored skin for 4 hr at 36 C had no consistent effect on the number of paranuclear vacuoles produced by SM in organ-cultured skin explants

Table 4: Hydroxyproline levels in organ-culture fluids from 1.0%-SM-exposed and control full-thickness human skin explants

	Day culture fluid was collected	SM-exposed skin OD at 560 nm	$MeCl_2$-exposed skin OD at 560 nm
Experiment #1 (preliminary)			
(no serum)	third day	0.060-0.070	0.100-0.110
Experiment #2 (with and without serum)			
With 10% fetal bovine serum	first day	0.050	0.060
	second day	0.030	0.055
	third day	0.025	0.075
Without fetal bovine serum	first day	0.040	0.065
	second day	0.030	0.125
	third day	0.030	0.175

An optical density (OD) of 0.500 at 560 nm represents 10 ug of hydroxyproline in our assay system (described in our Final Report of contract DAMD17-80-C-0102 (22). Experiments 1 and 2 used skin from different patients.

Table 5: Acid phosphatase activity in organ-culture fluids from 1.0%-SM-exposed and control full-thickness human skin explants

Day culture fluid was collected	Incubation time for acid phosphatase	Culture fluids incubated without skin explants	Culture fluids from SM-exposed skin explants	Culture fluids from $MeCl_2$-exposed skin explants	Amount of culture fluid in assay
		Optical density at 410 um			
First day	30 min	0.030-0.045	0.030-0.040	0.030-0.040	0.2 ml in 3.2 ml
Second day	"	"	0.035-0.045	0.030-0.045	
Third day	"	"	0.035-0.040	0.035-0.035	
First day	20 hr	0.101-0.104	0.095-0.110	0.095-0.100	0.5 ml in 3.5 ml
Second day	"	"	0.105-0.130	0.090-0.105	
Third day	"	"	0.100-0.110	0.095-0.100	

The optical density ranges of triplicate human skin explants are presented. An optical density reading of 0.100 was equivalent to 2.7 ug of nitrophenol in 3.0 ml read in the cuvette at 410 nm.

Incubation of the culture fluid without the skin explant provides a measure of the spontaneous hydrolysis of the nitrophenyl phosphate substrate during the incubation period. This control proved that the culture fluids from the human skin explants had minimal, if any, acid phosphatase activity.

Table 6: Trypsin-like enzymes in organ-culture fluids from 1.0%-SM-exposed and control full-thickness human skin explants

Substances added	Source of culture fluid	SM-exposed skin ug AFC released per 50 ul culture fluid	$MeCl_2$-exposed skin ug AFC released per 50 ul culture fluid
Plasminogen and aprotinin	A	6.5	6.4
	B	7.3	7.4
Plasminogen	A	10.3	10.2
	B	10.9	11.1
Aprotinin	A	0.18	0.25
	B	0.12	0.12
Nothing added	A	0.55	1.33
	B	0.46	0.18

The incubated mixture contained culture fluids (50 ul), 0.05 M TES buffer, pH 8.2 (800 ul), plasminogen 1.25 units in 0.9% NaCl (50 ul), and/or aprotinin 2.5 ug in 0.9% NaCl (50 ul), LGA-AFC substrate 20 mM in dimethyl formamide (50 ul), and sufficient 0.9% NaCl added to make a 1.0-ml final volume. TES designates N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (Sigma, Cat. No. T-1375).

First-day culture fluids from skin explants of two individuals (A and B) were assayed.

Table 7: DNase and RNase activity in organ-culture fluids from full-thickness human (and rabbit) skin explants exposed to 1.0% sulfur (or 5.0% nitrogen) mustard

Number of skin samples (n) in SM or $MeCl_2$ group	SM-exposed: change in optical density at 260 nm	$MeCl_2$-exposed: change in optical density at 260 nm	Ratio SM:$MeCl_2$ groups	Incubation time
	DNase -- Human skin			
# 1 (2)	0.210	0.145	1.4	2 hr
# 2 (2)	-0.080	-0.070	---	2 hr
# 3 (2)	-0.015	-0.005	---	2 hr
# 4 (2)	0.005	0.005	---	2 hr
# 5 (4)	0.045	0.035	---	2 hr
# 6 (6)	-0.008	0.000	---	2 hr
	DNase -- Rabbit skin			
# 1 (1)	0.190	0.180	---	2 hr
# 2 (1)	0.185	0.150	1.2	2 hr
# 3 (4)	0.185	0.150	1.2	2 hr
# 4 (2)	0.230	0.165	1.4	2 hr
# 5 (2)	0.025	0.025	---	2 hr
# 6 (2)	0.145	0.180	0.8	2 hr
# 7 (2)	0.110	0.000	high	2 hr
# 8 (2)	0.050	0.010	---	2 hr
	RNase -- Human skin			
# 1 (2)	0.490	0.175	2.8	2 hr
# 2 (6)	0.360	0.330	---	2 hr
# 3 (2)	0.335	0.330	---	2 hr

Table 7: DNase and RNase activity in organ-culture fluids from full-thickness human (and rabbit) skin explants exposed to 1.0% sulfur (or 5.0% nitrogen) mustard (continued)

In the experiments with rabbit skin, 5% nitrogen mustard (10 ul) was applied topically in vitro to the full-thickness skin explants, instead of 1% sulfur mustard (10 ul), because the latter could not be obtained at that time.

Table 8: Interleukin 1 activity of organ-culture fluids from from rabbit skin exposed in vitro to 1.0% SM with and without added serum and/or added macrophages

		SM		$MeCl_2$	
Incubation time	Macro-phages	No serum	Serum (10%)	No serum	Serum (10%)
3 hr (Exp. I)	-	4.6 ±0.5	5.0 ±0.2	5.7 ±0.8	12.3 ±1.1
	+	6.9 ±0.7	13.6 ±1.7	5.8 ±0.7	7.7 ±3.8
16 hr (Exp. I)	-	15.0 ±1.2	16.9 ±1.3	8.8 ±2.1	
	+	13.6 ±3.7	3.8 ±0.5	8.7 ±1.6	23.0 ±3.3
16 hr (Exp.II)	-	20.3 ±1.3	9.5 ±3.2	11.1 ±6.4	11.4 ±2.0
	+	10.3 ±6.0	19.2 ±2.5	28.8 ±4.1	12.2 ±0.8

Listed are the radioactive tritium disintegrations per min in hundreds at a 1:16 dilution of dialyzed culture fluid (see Materials and Methods). Values above 10.0 (i.e., 1000 dpm) can be considered to have IL-1 activity. The means and their standard errors from triplicate thymocyte cultures are presented.

Table 9: Interleukin 1 activity of organ-culture fluids from human skin exposed in vitro to 1.0% SM

Incubation time	Human skin specimen	SM	$MeCl_2$
3 hr	A	10.3 ±0.7	14.9 ±1.7
	B	12.9 ±2.6	9.1 ±1.2
20 hr	A	16.2 ±4.1	12.7 ±3.1
	B	9.1 ±0.6	10.0 ±1.6
48 hr	A	----	----
	B	13.2 ±1.4	13.3 ±1.0

Listed are the radioactive tritium disintegrations per min in hundreds at a 1:16 dilution of (dialyzed) culture fluid (see Materials and Methods). Values above 10.0 (i.e., 1000 dpm) can be considered to have IL-1 activity. The means (and their standard errors) from triplicate thymocyte cultures are presented.

Table 10: Comparison of <u>total</u> paranuclear vacuole counts made by two different investigators on the same tissue sections listed in Table 2

Skin Sample No. and type of skin	Days in storage at 4 C	Number of paranuclear vacuoles in a central 1.0-cm tissue section across the explant after 24-hr incubation at 36 C					
		Controls			0.2% SM		
		#1	#2	Ratio	#1	#2	Ratio
1							
Normal breast	1 day(s)	--	--	---	300	740	2.47
skin, black	4 "	12	15	1.25	740	1340	1.81
female, age 30	7 "	16	23	1.44	900	1070	1.19
2							
Normal breast	0 day(s)	11	59	5.36	1090	820	0.75
skin, white	5 "	70	91	1.30	630	510	0.81
female, age 27	9 "	36	41	1.14	1040	970	0.93
3	2 days	40	64	1.60	860	960	1.12
Normal breast	5 "	17	49	2.88	880	1110	1.27
skin,	8 "	15	25	1.67	990	1060	1.07
black female,	12 "	360	330	0.92	1010	1050	1.04
age 24	15 "	140	138	0.99	1020	900	0.88
4							
Normal leg skin,	3 days	4	31	--	410	280	0.68
white female,	6 "	7	30	--	720	420	0.58
age 79	10 "	8	85	--	520	250	0.48
5	1 day(s)	10	13	1.30	350	190	0.54
Normal leg skin,	4 "	15	28	1.87	460	380	0.83
white male,	8 "	132	172	1.30	410	230	0.56
age 72	15 "	159	230	1.45	390	290	0.74
		Mean: 1.75			Mean: 0.99		
		SE: ±0.29			SE: ±0.11		

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ACKNOWLEDGMENTS

The following scientists, of our laboratory, collaborated in this research project: Toru Rikimaru, Brian H. Schofield, Takafumi Yano, Masahiro Nakamura, and Peggy J. Pula. Ilse M. Harrop typed and edited the manuscript. The scientists from other laboratories who helped are mentioned in the text.

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